Original Article

Determination of Methotrexate in Biological Fluids and a Parenteral Injection Using Terbium-Sensitized Method

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Abstract

A new sensitive, simple and rapid method for determination of methotrexate (MTX) was developed based on quenching effects of MTX on the fluorescence intensity of Tb^{3+}-1,10-phenanthroline complex. The fluorescence intensity was measured with excitation wavelength of 300 nm and emission wavelength of 545 nm and the quenched fluorescence intensity is proportional to the concentration of MTX in Tris-HCl buffer solution with a pH of 7.0. The effects of pH, time, order of addition of the reagents, temperature and the concentrations of Tb^{3+}, buffer and 1,10-phenanthroline were investigated and optimized. The obtained linear range for the determination of MTX was 0.02-10 μg/mL. The detection limits (signal: noise = 3) was 0.015 μg/mL and the relative standard deviation for replicated determinations of 1 μg/mL of MTX was 1.9%. The proposed method is a simple, practical and relatively free from interference effects and was successfully applied to assess MTX in urine, serum and samples of an injection solution.

Keywords: Terbium-sensitized; 1,10-Phenanthroline; Methotrexate; Injection solution; Urine; Serum.

Introduction

Methotrexate (MTX) or (2S)-2-[[4-[(2,4-diaminopteridin-6-yl) methyl-methylamino] benzoyl] amino] pentanedioic acid, (Figure 1) is an antineoplastic which acts as an antimetabolite of folic acid. MTX is a tight-binding inhibitor of dihydrofolate reductase, the enzyme responsible for the regeneration of tetrahydrofolate (THF) from dihydrofolate. Inhibition of this enzyme induces cellular depletion of THF cofactors, including 5-methyl-THF, and thereby blocks several folate-related metabolic processes. These include synthesis of purines and salvage of homocysteine to methionine (1-3). MTX is used in low doses (7.5-25 mg/week) in the treatment of certain connective tissues diseases such as rheumatoid arthritis, lupus and scleroderma (2-5). MTX is also used in high doses (> 1 g/m² body surface) as an anti-cancer drug for treatment of certain forms of cancer (leukemia) (3, 6, 7). The efficacy of antifolate drugs is related to the extent of intra-cellular polyglutamation. In most cells, polyglutamation does not occur until the cell is exposed to 10^{-6} mol/L of MTX for at least 6 h (8, 9).

MTX is generally administered orally and

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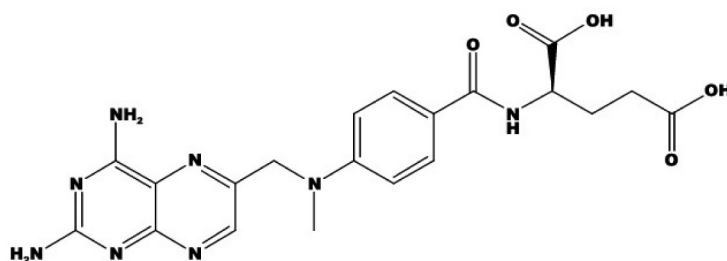
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Figure 1. Structure of methotrexate.

after absorption, most of drug is excreted unaltered in urine. Less than 10% of MTX dose is converted to 7-hydroxymethotrexate (7-OH-MTX) in the liver. Both MTX and 7-OH-MTX are primarily excreted in urine although a small portion is also excreted in the bile. MTX binds to proteins (35-50%), while 7-OH-MTX possesses 91-95% albumin-binding and high tissue distribution. The usual terminal serum half-life of MTX is approximately 7-10 h (4, 10, 11). MTX concentration in plasma and other biological fluids is determined to investigate its pharmacokinetics and also to predict and prevent its toxicity when administered in high doses via intra-venous infusion (8, 12). Therefore, the establishment of highly sensitive and simple methods for the determination of MTX is of great importance in the area of pharmaceutical sciences.

Several methods for determination of MTX in biological fluids have been reported including fluorescence polarization immunoassay (13, 14), enzymatic assay (15), enzyme-multiplied immunoassay (16), radioimmunoassay (17), HPLC methods (18-21), capillary zone electrophoresis (8, 22), fluorimetric methods (23, 24) and voltammetry (25) methods. Fluorimetric methods are based on the oxidation of MTX to pteridine. Carboxylic acid was applied to measure the plasma levels of MTX in cancer patients. Most of these methods exhibit higher sensitivities, but their selectivities are usually unsatisfactory. Although immunological methods are the most commonly used ones, they are time consuming, need great care and also more skilled analyst. Chromatographic methods have the advantage of separating with the minimum interference from enzymes but involve set up cost, a complex extraction, purification procedure, longer analysis time, requiring relatively large volumes (> 0.1 mL) and lack of sensitivity. The major problem of electroanalytical techniques is their poor sensitivity. These methods, however are rapid and sensitive, possess inherent difficulties that limit their applications.

Considering above mentioned limitations and in order to improve measurement method of low concentration of MTX in biological samples, a simple, rapid and sensitive spectrofluorimetric method based on quenching of terbium sensitized fluorescence (TSF) is proposed in this work. Lanthanides have found applications in different fields of biomolecular and medical research. When the rare-earth ions (in particular Tb^{3+} and Eu^{3+}) are chelated with ligand, their fluorescence can be dramatically enhanced. These chelates have unique fluorescence characteristics such as narrow spectral width, long fluorescence lifetime and large stokes shift. Therefore, terbium (III) chelates are often used as a fluorescence probe for determination of some substances, because of the high fluorescence quantum efficiency, based on the intensity fluorescence quenching or the enhancement of these chelates (26-29). Results of a previous study (30) demonstrated that Tb^{3+}-1,10-phenanthroline (phen) complex has a strong intrinsic fluorescence of Tb^{3+}. It was also found that its fluorescence could be quenched by MTX; therefore, a novel spectrofluorimetric method for determination of MTX could be developed.

Experimental

Reagents and solutions

All chemicals and solvents were of analytical reagent grade. Double distilled water was used throughout this work. A 10^{-2} mol/L terbium (III) solution was prepared by dissolving the
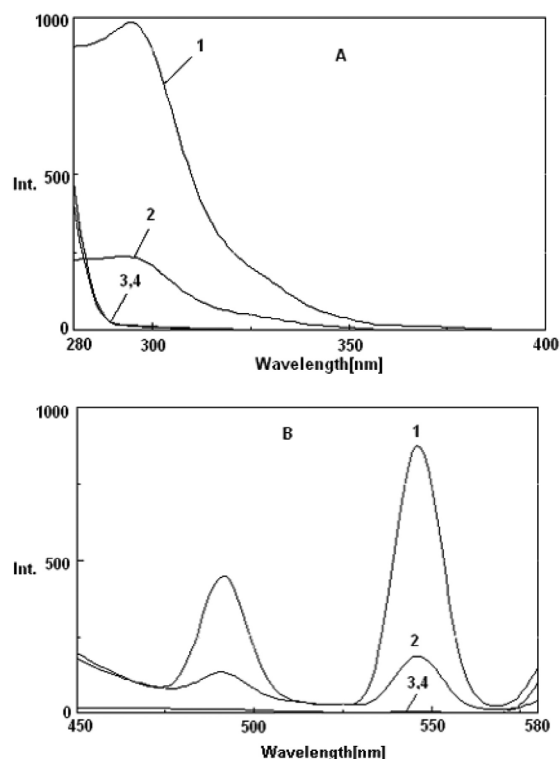
Figure 2. Terbium-sensitized fluorescence excitation (λ_{em} = 545 nm) (A) and emission (λ_{ex} = 300 nm) (B) spectra: (1) Tb^{3+}-phen; (2) Tb^{3+}-phen -MTX; (3) Tb-MTX; (4) Tb^3. Conditions: $[Tb^{3+}]$ = 2×10^{-4} mol/L, [MTX] = 2 µg/mL, [phen] = 10^{-4} M, (Tris-HCl = 0.01 M, pH = 7.0).

appropriate amount of terbium (III) chloride hexahydrate ($TbCl_3.6H_2O$) (Acros Organics, USA) in double distilled water and stored in polyethylene containers to avoid memory effects of terbium adsorbed on glass vessels.

MTX with a purity of more than 98% was purchased from Sigma. A 10000 µg/mL stock standard solution was prepared by dissolving 100.0 mg of MTX powder in approximately 2 mL of 0.1 mol/L sodium hydroxide and diluted to the mark in a 10 mL volumetric flask with 10% w/v NaCl solution. The stock solution was stored at -20°C. Appropriate MTX solutions of different concentrations (10, 100 and 1000 µg/mL) were prepared by dilution with distilled water, stored at 4°C and protected against light.

A 10^{-2} mol/L stock solution of 1,10-phenanthroline (Phen) (Fluka, Switzerland) was prepared in 10 mL ethanol and diluted to the mark in a 100 mL volumetric flask with double distilled water.

A 0.05 mol/L tris-(hydroxymethyl) aminomethane-hydrochloric acid (Tris- HCl)

buffer solution was prepared by dissolving a desired amount of Tris-base (Merck, Germany) in 90 mL of water, adjusting the pH to 7.0 with HCl and making up the volume to 100 mL with water. Working standard solutions were prepared daily by successive dilution of the stock standard with water. MTX (50 mg/5 mL) injections were purchased from a local pharmacy store with the trademark of Ebetrex.

Apparatus

Fluorescence spectra and intensity measurements were performed using a Jasco FP-750 spectrofluotimetry (Japan) equipped with a 150 W xenon lamp, using 1.0 cm quartz cells. All measurements were performed at 25 ± 0.1°C using a Peltier thermostatted cell holder (Jasco, Japan). The pH of solutions was measured with Metrohm model 744 pH meter (Switzerland).

Experimental procedure

For the determination of MTX, the analytical procedure used to construct the calibration graph was as follow: 1 mL of 1×10^{-3} mol/L Tb^{3+} solution, 1 mL of 0.05 mol/L Tris-HCl buffer (pH 7.0) solution, 0.25 mL of 1×10^{-3} mol/L Phen solution and aliquots of working MTX solutions (10 and 100 µg/mL) were added to 5 mL volumetric flasks. The mixture was then diluted to the mark with distilled water. The final MTX concentration was in the range of 0.02-10 µg/mL. The solutions were thermostatted at 25 ± 0.1°C and the fluorescence intensities of the solutions and blank were measured at 545 nm using an excitation wavelength of 300 nm immediately after the addition of reagents (during 2 min). Both emission and excitation slits were set at 10 nm. The quenched fluorescence intensity of Tb^{3+}-Phen by MTX was represented as I_0/I_f in which I_f and I_0 were the intensities of the systems with and without MTX, respectively.

Sample preparation

The proposed procedure for the determination of MTX was applied to a parenteral formulation (50 mg/5 mL, Ebetrex®, Unterach, Austria). In addition, MTX in human urine and serum was determined. For determination of MTX in an injection solution, the diluted samples were used.
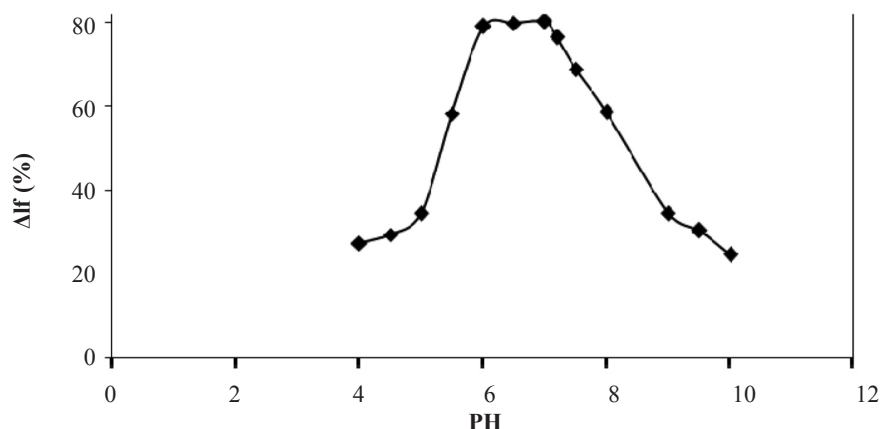
Figure 3. Effect of pH on the quenched fluorescence intensity Tb^{3+}-phen -MTX system. Conditions: [phen], 2×10^{-4} mol/L; [Tb^{3+}], 10^{-4} mol/L; [MTX], 2 µg/mL. ΔI_f (%) = $(I_0 - I_f)/I_0 \times 100$ in which I_f and I_0 were the intensities of the systems with and without MTX, respectively.

Experimental procedure for the determination of MTX in serum sample

The analytical procedure used to construct the calibration graph was as follow: an aliquot of 100 µL serum with convenient amounts of MTX stock solutions (1000 and 10000 µg/mL) was added to 1 mL micro tubes. Then, samples were vortexed for 30 sec and after 15 min, 400 µL acetonitrile was added to remove proteins. Deproteinization was carried out by vortexing for 5 min and centrifugating for 15 min at 10000 rpm. Afterward, 0.5 mL of the supernatant was pipetted to a 10 mL volumetric flask and 0.5 mL was used to analyze as explained for the standard solution of MTX. MTX concentrations in final solutions after the dilution were in the range of 0.05-8.3 µg/mL. The fluorescence intensity of solutions and free drug serum (blank) was measured at 545 nm using an excitation wavelength of 300 nm. The recovery assay was carried out using the same procedure by addition of known amounts of MTX. Real serum samples taken from patients were also determined.

Experimental procedure for the determination of MTX in human urine sample

The analytical procedure used to construct the calibration graph was as follow: an aliquot of 50 µL urine with convenient amounts of MTX stock solutions (100, 1000 and 10000 µg/mL) was spiked and diluted to 10 mL with distilled water. Then, an aliquot 0.1 mL of the diluted samples in a 5 mL volumetric flask was

measured as explained for standard MTX (1000 fold). The MTX concentration in final solutions after diluting is in range of 0.02-10 µg/mL. The fluorescence intensity of solutions and free drug (blank) urine was measured at 545 nm using an excitation wavelength of 300 nm. The recovery assay was carried out using the same procedure by addition of known amounts of MTX.

Results and Discussion

Fluorescence spectra

Fluorescence excitation and emission spectra of (1) Tb^{3+}-Phen, (2) Tb^{3+}-Phen-MTX, (3) Tb^{3+}-MTX and (4) Tb^{3+} systems are shown in Figure 2. The characteristic peak of Tb^{3+}-Phen was observed with two emission peaks at 545 nm and 490 nm. The fluorescence spectrum of Tb^{3+}-Phen-MTX system was similar to that of Tb^{3+}-Phen; however, the fluorescence intensity was decreased by MTX. These results indicated that there was an interaction between MTX and Tb^{3+}-Phen. Therefore, the Tb^{3+}-Phen-MTX system was utilized in the assays of MTX. Since the quenched fluorescence intensity at 545 nm was higher, this wavelength was chosen to detect the fluorescence intensities throughout all the experiments.

Factors affecting the fluorescence intensity of the system

Effect of pH and concentration of buffer
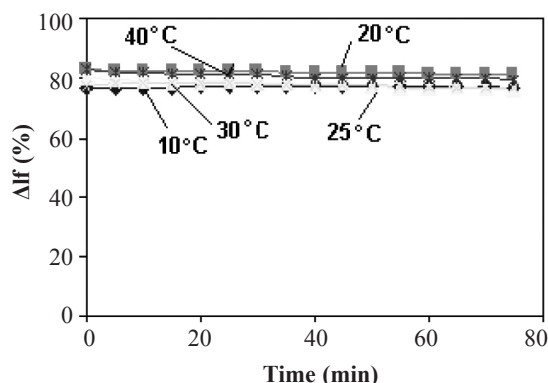
The effect of pH on the quenched

Figure 4. Effect of time on the fluorescence intensity and stability of Tb-phen-MTX system in different temperature. Conditions: [phen], 2×10^{-4} mol/L; [Tb^{3+}], 10^{-4} mol/L; [MTX], 2 μg/mL; (Tris-HCl = 0.01 M, pH = 7.0).

Figure 5. Effect of Tb^{3+} concentration on the quenched Fluorescence intensity. Conditions: [phen], 2×10^{-4} mol/L; [MTX], 2 μg/mL, (Tris-HCl, 0.01 mol/L, pH = 7.0).

fluorescence intensity (ΔI_f (%)) of the Tb^{3+}-Phen-MTX system in the pH range of 4.5-10.0 (Figure 3) was investigated. As it is obvious in Figure 3, the decreased fluorescence intensity (ΔI_f (%)) of Tb^{3+}–Phen complex is strongly dependent on pH and is reached to a maximum value between pH 6.8 and 7.2. Thus, pH of 7.0 was selected as the optimum pH. This pH was adjusted by Tris buffer solution. Experiments indicated that the chemical nature of the buffer has also considerable effects on the quenched fluorescence intensity. The results showed that 1 mL of Tris-HCl (0.05 mol/L, pH = 7.0) in a final 5 mL was the most suitable concentration. Tris buffer also increases the obtained signal, probably due to its penetrating the coordination sphere of the chelate, giving rise to a synergistic effect.

Effect of time and temperature

Effect of time and different temperatures on the fluorescence intensity (ΔI_f (%)) of Tb^{3+}-Phen and Tb^{3+}-Phen-MTX systems was studied within 80 min (Figure 4). As shown in Figure 4, in all temperatures, the fluorescence intensity of Tb^{3+}-Phen and Tb^{3+}-Phen-MTX systems is approximately constant. Therefore, the quenched fluorescence intensity of the systems is very stable and determination can be carried out immediately after the addition of all regents. The other results also showed that the quenched fluorescence intensity of the systems remain stable for more than 12 h.
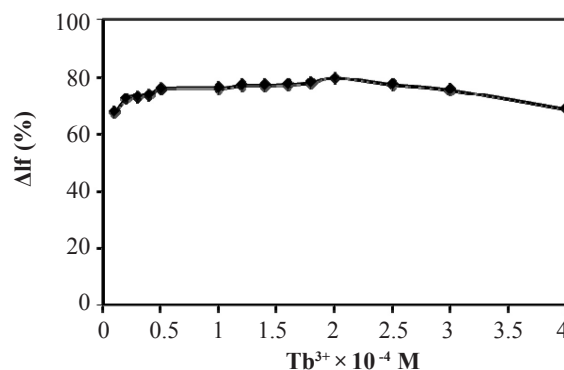
Effect of concentration of terbium

The effect of the concentration of Tb^{3+} on the quenched fluorescence intensity (ΔI_f (%)) of Tb^{3+}-Phen-MTX system in constant concentration of Phen was studied and results are shown in Figure 5. It can be seen that ΔI_f (%) has been the highest when the concentration of Tb^{3+} in the mixture was 2.0×10^{-4} mol/L. Therefore, the concentration of Tb^{3+} in the mixture was chosen at 2.0×10^{-4} mol/L for further research.

Effect of concentration of phen

The effect of Phen concentration on the fluorescence intensity (ΔI_f (%)) of Tb^{3+}-Phen-MTX system was studied and as the results show in Figure 6, it was found that ΔI_f (%) of Tb^{3+}-Phen-MTX system reached to a maximum value when the concentration of Phen was 0.5×10^{-4} mol/L. Therefore, this value was used for further study.

Effect of addition order

Finally, the effect of addition order was tested. For this purpose, a series of solutions with different addition orders of reagents and their corresponding blank solutions were measured at $\lambda_{ex}/\lambda_{em}$ = 300 nm/545 nm. Based on the results and considering the system stability along with the quenched fluorescence intensity enhancement, the order of Tb^{3+}, Tris-HCl, Phen and MTX was the best and followed in further investigations.
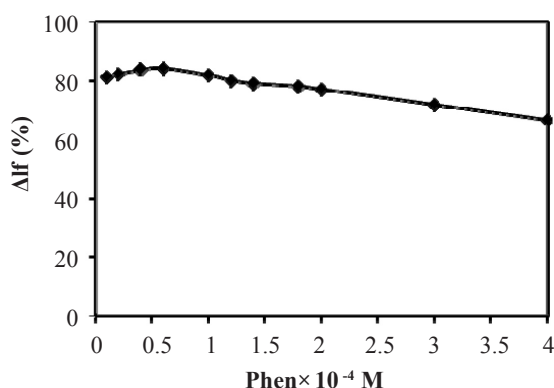
Figure 6. Effect of phen concentration on the quenched fluorescence intensity. Conditions: [Tb^{3+}], 2×10^{-4} mol/L; [MTX], 2 μg/mL; (Tris-HCl, 0.01 mol/L, pH = 7.0).

Concentration ranges and calibration graphs

By using the optimized conditions described above, a spectrofluorimetric method was developed for the determination of MTX in the biological samples. The measurements are based on the Stern-Volmer equations (31, 32) which describes dynamic or collisional quenching requiring contact between the excited luminophore and the quencher Q, as follows:

$$I_0/I_f = 1 + K_q \tau_0 [Q]$$

In this equation, I_0 and I_f are the fluorescence intensities in the absence and the presence of a quencher, respectively; K_q is the quenching rate constant (Lmol^{-1}s^{-1}) and τ_0 is the fluorescence lifetime(s) in the absence of the quencher. The equation shows that a plot of I_0/I_f versus the quencher concentration should give a straight line usable for the determination of the quencher. A high value of K_q leads to a low limit of detection for the quencher. The data showed that there is linearity between the MTX concentration and the quenched fluorescence intensity of the Tb^{3+}-Phen system.

The calibration graph (the plot of I_0/I_f versus the MTX concentration) (n = 10) was found to be linear in the range of 0.02 to 10 μg/mL for MTX and its equation was as follows:

$$I_0/I_f = 1.0969 \, C + 1.0176 \; (r = 0.999)$$

Where I_0/I_f is the quenched fluorescence intensity of Tb^{3+}-Phen by MTX and C is the concentration of MTX expressed in μg/mL. Standard deviations (SD) for the slope and intercept of the calibration graph were ± 0.0037 and ± 0.0025, respectively.

The limit of detection (LOD) and the limit of quantification (LQD)

In accordance to ICH definition (33), the limit of detection was calculated as $3S_b/m$ (where S_b is standard deviation of the blank and m is slope of the calibration graph). Using this formula, LOD was found to be 0.015 μg/mL. The limit of quantification was defined as $10S_b/m$ and found as 0.052 μg/mL.

Precision and accuracy

In order to investigate the precision of the proposed method (repeatability), series of six solutions of 0.05, 1.0 and 4.0 μg/mL of MTX were measured on the same day. By applying the ICH definition (36), the relative standard deviation (RSD) for six analyses was 0.5, 1.9 and 0.4%, respectively. To assess the day-to-day precision (intermediate precision), repeated analyses of 1.0 and 4.0 μg/mL of MTX (six analyses) were performed over one month and interday RSD were 2.9 and 0.8%, respectively.

Specificity and interference study

For the possible analytical application of the proposed method, a systematic study of the effects of coexisting substances that was likely in biological samples on the reduced fluorescence intensity ($\Delta I_f\%$) was carried out under the optimal conditions. The effects of these species were evaluated by the addition of increasing concentration of various species to a fixed amount of MTX (2 μg/mL), under the same experimental conditions, until variation greater than 10% in analytical intensity was achieved. The tolerance levels of various interferents (*e.g.* ions, amino acids, proteins and saccharides) are summarized in Table 1. Careful examination of Table 1 revealed that the most substances in low amounts were found to show small effects on the determination of MTX under permission of ± 10% relative error. In practice, through the dilution of urine samples up to 1000-fold and deproteinization of serum samples and with applying standard addition method, interference-

Table 1. Effects of common interferents on the determination of 2 μg/mL MTX.

Coexisting substance	Ratio of Coexisting substance to MTX	$\Delta I_F\%$
K^+ (Cl⁻)	1:75	0.8
Na^+ (Cl⁻)	1:150	2.4
Ca^{2+} (Cl⁻)	1:75	4.4
Ba^{2+} (Cl⁻)	1:75	-1.1
Al^{3+} (Cl⁻)	1:0.25	-7.8
Cr^{3+} (Cl⁻)	1:0.02	-3.5
Au^{3+} (Cl⁻)	1:0.20	4.5
Zn^{2+} (Cl⁻)	1:0.4	2.6
Mn^{2+} (Cl⁻)	1:0.4	3.9
Ni^{2+} (Cl⁻)	1:0.2	-1.3
Cu^{2+} (Cl⁻)	1:0.15	4.1
Cd^{2+} (Cl⁻)	1:0.25	2.3
Co^{2+} (Cl⁻)	1:0.2	0.9
L-Alanine	1:75	3.6
L-Cysteine	1:25	-5.0
Tryptophane	1:70	3.5
Glycine	1:25	2.7
L-Leucine	1:125	-5.4
Tyrosine	1:25	0.8
Uric acid	1:0.025	-1.9
Sacarose	1:12.5	2.6
Glucose	1:12.5	-2.9
Bovine serum albumin	1:0.20	-3.7
Folic acid	1:0.25	-3.8
Folinic acid	0.2:1	-4.5

free determination of MTX is possible. Hence, the selectivity achieved by the proposed method is good and it is possible to determine the MTX. Folic acid or folinic acid were co-administered with MTX and their interferences were also investigated and the tolerance ratios were 0.3 and 0.2, respectively.

Analytical applications
Determination of MTX in an injection solution
The developed method was applied to the determination of MTX in an injection solution and the results were shown in Table 2. One injection (containing 50 mg/5 mL) was directly diluted to 100 mL and then analyzed using the standard calibration method. The concentration

of MTX in the injection solution was found as 51.5 mg/5 mL.

Determination of MTX in urine samples
To demonstrate the usefulness of the proposed method, several aliquots of MTX were added to urine. The standard addition method was used for MTX determinations due to the possible matrix effect from urine. For the assays of MTX in samples of urine, the fresh samples must be diluted appropriately within the linear ranges of determination (1000 fold). A portion of these sample solutions was analyzed through the developed method, using the standard calibration method. In these conditions, there is no interference in determination of MTX and this method can be utilized for direct determination of MTX after dilution.

The precision and accuracy of the method were investigated by adding different concentrations of MTX. Recoveries obtained for different concentrations of MTX spiked to urine samples were between 98.4% and 104.8% (Table 2) with the RSDs less than 3%. Inter day RSD values for concentrations of 200 and 40 μg/mL were 0.7% and 1.5%, respectively. Therefore, the proposed method was easy to perform for the direct determination of MTX without requiring any pretreatment step.

Determination of MTX in patient serum samples
The developed method was applied to the determination of MTX in human serum samples after a prior protein precipitation using acetonitrile with serum (acetonitrile ratio of 1 : 2). It should be noted that the dilution (1000 fold) of serum samples following the protein precipitation step is necessary. The dilution diminishes the high background fluorescence measured for blank serum sample.

The precision and accuracy of the method were measured by the added-found procedure. Spiked serum samples were prepared with addition of different concentrations of MTX, around therapeutic concentrations in human serum (50-300 μg/mL) and analyzed on different days for measuring inter-day precision (using the calibration graph on each day). Intra-day precision was obtained by replicate analyses

Table 2. Determination of MTX in urine samples.

Added (μg/mL)	Found* (μg/mL)	Recovery (%)	RSD (%)
25.0	26.0 ± 0.4	104.8 ± 1.7	1.5
40.0	40.5 ± 0.4	101.2 ± 1.2	1.0
100.0	99.5 ± 4.1	98.4 ± 1.9	4.2
200.0	209.5 ± 0.7	104.7 ± 0.4	0.3
300.0	314.5 ± 8.0	104.7 ± 2.7	2.5

* Mean of five observations ± standard deviation.

of the serum samples on the same day. The data of Table 3 demonstrate an acceptable precision and accuracy over the investigated concentration range. Recoveries obtained for different concentrations of MTX in spiked serum samples were between 97.5% and 110.5% (Table 3) with the RSDs less than 5%. Therefore, the method is appropriate for determination of MTX in serum and meets the requirement of rapidity for routine use in high dose MTX therapy. Also, the advantage of a low-cost and easily handling instrument should be taken into consideration.

The regression equation (n = 21) was $I_0/I_f = 0.7918$ C + 0.9953 (r = 0.999) and the standard deviations (SD) for the slope and intercept of the calibration graph were ± 0.0012 and ± 0.0078, respectively. Results of analysis of a real serum sample showed that MTX level was 2244 mg/L (sample was diluted up to 4000 times to be in the linear range of the calibration graph).

Conclusion

A new sensitive method for simple and rapid determination of MTX was developed for the determination of trace amounts of MTX. Phen forms a binary complex with Tb^{3+}, which emits at 545 nm. MTX can remarkably reduce its fluorescence intensity at this wavelength. The reduction in the fluorescence intensity of the Tb^{3+}-Phen probe is proportional to the concentration of MTX. This method was very simple, precise and rapid and also was easily applied to the determination of MTX in real samples with good reproducibility and allowed the interference-free determination of MTX in real samples. The presented method is not very selective in comparison with the HPLC methods, but it is fast and requires small volume of serum or urine samples for determination of MTX and besides, no sample pre-treatment is required.

Table 3. Recovery of MTX in serum samples.

	Added (μg/mL)	Found * (μg/mL)	Recovery (%)	RSD (%)
	50.0	55.2 ± 1.8	110.5 ± 3.3	3.3
	100.0	106.3 ± 2.6	106.7 ± 2.6	2.4
Intra-day	250.0	254.5 ± 0.7	98.2 ± 0.30	0.30
	300.0	314.5 ± 4.0	104.9 ± 1.3	1.3
	50.0	53.2 ± 2.4	106.4 ± 4.8	4.5
Inter-day	100.0	106.4 ± 1.4	106.4 ± 1.4	1.3
	250.0	243.7 ± 3.7	97.5 ± 1.5	1.5
	300.0	323.0 ± 7.1	107.7 ± 2.4	2.2

*Mean of five observations ± standard deviation.

References

(1) Sabry SM, Abdel-Hady M, Elsayed M, Fahmy OT and Maher HM. Study of stability of methotrexate in acidic solution spectrofluorimetric determination of methotrexate in pharmaceutical preparations through acid-catalyzed degradation reaction. *J. Pharm. Biomed. Anal.* (2003) 32: 409-423.

(2) Bleyer WA. The clinical pharmacology of methotrexate: New applications of an old drug. *Cancer* (1978) 41: 36-51.

(3) Moghbel A, Zand-Moghaddamb A, Rezaee S and Pedram M. High-performance liquid chromatography determination of methotrexate in plasma. *Iranian J. Pharm. Res.* (2003) 2: 149-152.

(4) Ranganathan P and McLepd HL. Methotrexate pharmacogenetics. *Arthritis Rheum.* (2006) 54: 1366-1377.

(5) Pavy S, Constantin A, Pham T, Gossec L, Maillefert JF, Cantagrel A, Combe B, Flipo RM, Goupille P, Le Loët X, Mariette X, Puéchal X, Schaeverbeke T, Sibilia J, Tebib J, Wendling Dand Dougados M. Methotrexate therapy for rheumatoid arthritis: Clinical practice guidelines based on published evidence and expert opinion. *Joint Bone Spine* (2006) 73: 388-395.

(6) Vahid Roudsari F, Ayati S, Saghafy N and Shakeri M. Misoprostol alone or in combination with methotrexate for termination of pregnancy at first trimester. *Iranian J. Pharm. Res.* (2010) 9: 89-94.

(7) Levitt M, Mosher MB, DeConti RC, Farber LR, Skeel RT, Marsh JC, Mitchell MS, Papac RJ, Thomas ED and Bertino JR. Improved therapeutic index of methotrexate with «leucovorin rescue». *Cancer Res.* (1973) 33: 1729.

(8) Rodrıguez Flores J, Castaneda Penalvo G, Espinosa Mansilla A and Rodrıguez Gomez MG. Capillary electrophoretic determination of methotrexate, leucovorin and folic acid in human urine. *J. Chromatogr. B* (2005) 819: 141–147.

(9) Gao L, Wu Y, Liu J and Ye B. Anodic voltammetric behaviors of methotrexate at a glassy carbon electrode and its determination in spiked human urine. *J. Electroanal. Chem.* (2007) 610: 131–136.

(10) Swierkot J and Szechinski J. Methotrexate in rheumatoid arthritis. *Pharmacol. Rep.* (2006) 58: 473-492.

(11) Henghe Tian MD, Bruce N and Cronstein MD. Understanding the mechanisms of action of methotrexate. *Bull NYU Hosp. Joint Dis.* (2007) 65: 168-173.

(12) Fleisher M. Antifolate analogs: Mechanism of action, analytical methodology, and clinical efficacy. *Ther. Drug Monit.* (1993) 15: 521-529.

(13) Pesce MA and Bodourian SH. Evaluation of a fluorescence polarization immunoassay procedure for quantitation of methotrexate. *Ther. Drug Monit.* (1986) 8: 115-121.

(14) Hayashi H, Fujimaki C, Tsuboi S, Matsuyama T, Daimon T and Itoh K. Application of fluorescence polarization immunoassay for determination of methotrexate-poltglutamates in rheumatoid arthirits patients. *Tohoku J. Exp. Med.* (2008) 215: 95-101.

(15) Falk LC, Clark DR, Kalman SM and Long TF. Enzymatic assay for methotrexate in serum and cerebrospinal fluid. *Clin. Chem.* (1976) 22: 785-788.

(16) Buice RG, Evans WE, Karas J, Nicholas CA, Sidhu P, Straughn AB, Meyer MC and Crom WR. Evaluation of enzyme immunoassay, radioassay, and radioimmunoassay of serum methotrexate, as compared with liquid chromatography. *Clin. Chem.* (1980) 26: 92-98.

(17) Raso V and Schereider R. A rapid and specific radioimmunoassay for methotrexate. *Cancer Res.* (1975) 35: 1407-1410.

(18) McCruddena EA and Tettb SE. Improved high-performance liquid chromatography determination of methotrexate and its major metabolite in plasma using a poly(styrene-divinylbenzene) column. *J. Chromatogr. B* (1999) 721: 87-92.

(19) Duran Meras I, Espinosa Mansilla A and Rodriguez Gomez M. Determination of methotrexate, several pteridines, and creatinine in human urine, previous oxidation with potassium permanganate, using HPLC with photometric and fluorimetric serial detection. *Anal. Biochem.* (2005) 346: 201-209.

(20) Komeilizadeh H, Foroutan SM, Zarghi A and Shafaati A. Quantification of Polyethylene Glycol Esters of Methotrexate and Determination of Their Partition Coefficients by Validated HPLC Methods. *Iranian J. Pharm. Res.* (2009) 8: 27-31.

(21) Li H, Luo W, Zengb Q, Lin Z, Luo H and Zhang Y. Method for the determination of blood methotrexate by high performance liquid chromatography with online post-column electrochemical oxidation and fluorescence detection. *J. Chromatogr. B* (2007) 845: 164-168.

(22) Kuo CU, Wu HL, Kou HS, Chiou SS, Wu DC and Wu SM. Simultaneous determination of methotrexate and its eight metabolites in human whole blood by capillary zone electrophoresis. *J. Chromatogr. A* (2003) 1014: 93-101.

(23) Chakrabarti SG and Bernstein IA. A simplified fluorometric method for determination of plasma methotrexate. *Clin. Chem.* (1969) 15: 1157–1161.

(24) Chen S, Zhang Z, He D, Hu Y, Zheng H and He C. Flow-injection-electrochemical oxidation fluorimetry for determination of methotrexate. *Luminescence* (2007) 22: 338-342.

(25) Wang F, Wu Y, Liu J and Ye B. DNA Langmuir-Blodgett modified glassy carbon electrode as voltammetric sensor for determinate of methotrexate. *Electrochim. Acta* (2009) 54: 1408-1413.

(26) Tong C, Hu Z and Liu W. Sensitive determination of DNA based on the interaction between norfloxacin-Tb^{3+} complex and DNA. *J. Agric. Food Chem.* (2005) 53: 6207-6212.

(27) Tong CL, Xiang GH, Huang DJ and Liu WP. Determination of ofloxacin by the terbium(III) ion

fluorescence probe sensitized by the surfactant. *Chin. J. Anal. Chem.* (2004) 32: 619-621.

(28) Lis S. Luminescence spectroscopy of lanthanide (III) ions in solution. *J. Alloys Compd.* (2002) 341: 45-50.

(29) Georges J. Lanthanide-sensitized luminescence and applications to the determination of organic analytes. *Analyst* (1993) 118: 1481-1486.

(30) Kang JG, Kim TJ, Kang HJ and Kang SK. Crystal structures and luminescence properties of [Eu(ODA)·(phen)·4H$_2$O]$^+$, [Tb(ODA)·(phen)·4H$_2$O]$^+$ and [Tb(ODA)$_3$]$^{3-}$ (ODA: oxydiacetate, phen: 1,10-phenanthroline). *J. Photochem. Photobiol. A: Chem.* (2005) 174: 28-37.

(31) Sasirekha V, Umadevi M and Ramakrishnan V. Solvatochromic study of 1,2-dihydroxyanthraquinone in neat and binary solvent mixtures. *Spectrochim. Acta A* (2008) 69: 148-155.

(32) Thipperudrappa J, Biradar DS, Lagare MT, Hanagodimath SM, Inamdar SR and Kadadevaramath JS. Fluorescence quenching of BPBD by aniline in benzene-acetonitrile mixtures. *J. Photochem. Photobiol. A: Chem.* (2006) 177: 89-93.

(33) ICH Harmonised Tripartite Guideline, validation of analytical procedures: text and methodology, Q2 (R1). Website. http://www.ich.org /[4 May 2010].

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